ICBR-NEXTGEN DNA SEQUENCING
PacBio sample requirements for library construction

(Solution concentration: 30-50 ng/ul as quantitated by fluorescence … QUBIT)

Library Size and Type		Amount required	
DNA	20-30 Kb	DNA	20 µg
	10-20 Kb		10-15 µg
	5 Kb		5.0 µg
	2 Kb		1.5-2.0 µg
	1 Kb		1.0-1.5 µg
RNA (IsoSeq)	<3kb and >3kb fractions	RNA	2 µg total RNA
*Amplicons	<1 kb	*Amplicons	300 ng total
	1-3 kb		600 ng total
	3-10 kb		1.2 µg total
Bacterial gDNA for multiplexing	10 kb	Up to 16 samples per SEQUEL run	1.5 µg per sample

*The input amount for each amplicon depends on the multiplex level and amplicon size. To determine the input per sample, divide the total amount required for a given amplicon size by the number of samples to be pooled for sequencing in multiplex. The maximum volume per amplicon sample is 4 µl.

PacBio sample requirements when submitting pre-constructed libraries

(Solution concentration: 5-10 ng/ul as quantitated by fluorescence … QUBIT)

Library Size and Type		Amount required per 1-10 SMRT cell runs
DNA	20-30 Kb	200 ng (10 ng/ul)
	10-20 Kb	100 ng
	5 Kb	60 ng
	2 Kb	40 ng
	1 Kb	30 ng
IsoSeq (cDNA)	Pool of <3k and >3kb fractions	
Amplicons	250-500 bp (amplicons)	20 ng

The Pacific Biosciences® genomic DNA library preparation process does not utilize amplification techniques and resulting library molecules are directly used as templates for the sequencing process. As such, the quality of the DNA starting material will be directly reflected in the extent of sequencing success or failure. Any unrepaired or irreversible DNA damage present in the input material (e.g., interstrand crosslinks, nicks, etc.) will result in impaired performance in the system. High-quality, high-molecular-weight genomic DNA is imperative for obtaining long read lengths and optimal sequencing performance.

Required quantitation method: For DNA we recommend pico green or equivalent fluorometric method (e.g., QUBIT), NOT nanodrop or other absorbance-based method). For RNA, please use either ribo-green or QUBIT.

DNA quality: Generally DNA should be high molecular weight (significantly higher than the desired insert size in the library) without any smear of degradation products. For plant or soil DNA we recommend a final clean-up using the MOBIO powerclean kit (see below). DNA prep must be RNase-treated.

RNA quality: Good quality, intact RNA is essential. RIN must ≥8.0 as indicated by the Agilent Bioanalyzer. The RNA prep must be DNase-treated.

Buffer and concentration: DNA or RNA should be in 10 mM Tris, pH 7.5-8.0 at a minimum concentration of 30-50ng/ul.

Ensure that your DNA sample:

- Is double-stranded. Single-stranded DNA will not ligate to the adaptors in the library preparation process and can interfere with quantitation and polymerase binding at the sequencing step.
- Has undergone a minimum of freeze-thaw cycles.
- Has not been exposed to high temperatures (> 65ºC for 1 hour can cause a detectable decrease in sequence quality), pH extremes (< 6 or > 9).
- Has not been exposed to intercalating fluorescent dyes or ultraviolet radiation. If purifying DNA from a gel fragment, DO NOT use ethidium bromide for staining. We recommend SYBR Safe DNA Gel Stain with visualization on a blue light box (https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-stains/sybr-safe.html). UV will damage. Even a few seconds of UV irradiation appears to render DNA non-sequenceable.
- Has an OD260/280 ratio of approximately 1.8 to 2.0 (Nanodrop). Also, the OD260/230 ratio should higher than 2.0
- Does not contain insoluble material.
- Does not contain RNA contamination.
- Does not contain chelating agents (ie. EDTA), divalent metal cations (i.e.,Mg^{2+}), denaturants (Guanidinium salts, Phenol), or detergents (SDS,Triton-X100).
- Does not contain carryover contamination from the starting organism/tissue (heme, humic acid, polysaccharides, polyphenols, etc.). Some DNA isolation procedures might contain some contaminant(s) that can affect the quality of the run. We recommend performing addition clean-up steps using the PowerClean DNA Clean-up kit by MoBio.

- Is quantified by a fluorometric method (QUBIT or PicoGreen). Nanodrop (absorbance) is not adequate for quantification, although it does provide valuable information about sample's purity.

The MoBio kit information is listed here.

http://www.mobio.com/secondary_dna_clean-up/powerclean-dna-clean-up-kit.html
MoBio PowerClean DNA Cleanup Kit (# 12877-50)
New equivalent: MoBio PowerClean Pro Cleanup kit (# 12997-50)
Or QIAGEN DNeasy PowerClean Cleanup kit (# 12877-50)

Typically you will have 30%-50% recovery yield post MoBio kit treatment.

Before DNA extraction (recommended by PacBio):

a. Avoid incubation in complex or rich media
b. Harvesting from several cultures rather than a single, high-density culture during early- to mid-logarithmic growth phase is preferred.
c. Extraction of small volumes is preferred over large volumes to avoid accumulating high concentrations of potentially inhibiting secondary components.

Options for DNA Extraction: (no official endorsement from PacBio)

- Qiagen® MagAttract® HMW lit (100-200 kb)
- Ommiprep
- Qiagen Genomic-tip kit (50-100 kb)
- Qiagen Gentra® Puregene® kit (100-200 kb)
- Phenol-chloroform extraction (typically not recommended, but use only attached shared protocol if needed). Ensure phenol is fresh and not oxidized; use within three months of opening of reagent bottle.

Special recommendations for Plant RNA and DNA preparations

Pacbio library constructions (DNA or RNA) are so much more demanding than for other platforms, in terms of purity and quality. Phenolics and polysaccharides are a recurrent problem with plant DNA and RNA preps. The level of these substances varies greatly between tissues, and care must be taken to minimize their presence, as they are inhibitors of many downstream, enzymatic procedures.

At our facility, the MOBIO PowerClean has occasionally helped us in rescueing libraries that have failed to sequence. However, this reagent by no means has been a panacea.

Isolating good quality DNA and RNA from plants can be particularly difficult. While there are many recipes and methods that can be found in the literature, most may get rid of some but not all of the polysaccharides that may be present in the sample. Likewise, phenol-chloroform procedures can result in preps that are RT-inhibiting, etc. We don't endorse any particular

provider. However, in our lab we have found the Clontech-Takara (the provider of the the SMRTR full-length transcript cDNA synthesis reagents in the IsoSeq procedure) to be particularly robust, especially for RNA samples intended for use in the IsoSeq procedure.

Problem	Recommended product	Cat # (Clontech)
Plant RNA isolation (general)	NucleoSpin Plant Total RNA purification	740949.20
Polysaccharide removal	a) High-Salt Solution for precipitation b) Fruitmate for RNA purification	a) 9193 b) 9192
Cleaning Phenol-chloroform preps	NucleoSpin RNA Clean-up	740948.10
Plant DNA isolation (general)	NucleoSpin Plant Total DNA purification	9194

If using the InVitrogen TRIzol procedure for RNA isolation, it is recommended that it be used in combination with the PureLink RNA Mini Kit for increased purity (Cat #s 12183018A and 12183025).

Sample Shipping information:

We typically recommend samples be shipped in dry ice. Microcentrifuge tubes should be firmly capped, further secured with saran-wrap, and placed inside 50 ml conical tubes for shipment.

If preferred samples can be lyophilized and sent by "regular" mail. If you need to declare anything, please indicate that there are "non-toxic nucleotides samples".

ICBR CORE FACILITY INFORMATION	
Shipping Address	Request Information: Contact
NextGenSeq Laboratory	Email: ICBR-NextGenseq@ad.ufl.edu
CGRC Room 178, Univ. of Florida	Tel: (352) 273-8050
2033 Mowry Road	Fax: (352) 273-8070
Gainesville, FL 32610	
For Bioinformatics services, please contact ICBR-Bioinformatics@ad.ufl.edu	